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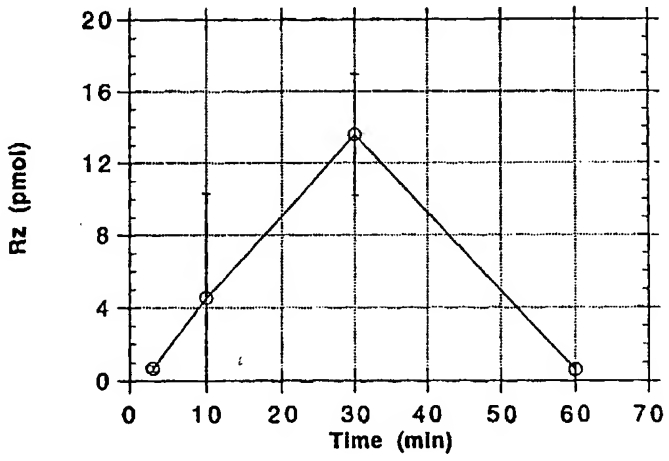

(57) Abstract

Pharmaceutical composition comprising polyacrylic acid and a therapeutically effective amount of a nucleic acid.

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DESCRIPTION

Ocular Delivery of Nucleic Acid

Background of the Invention

This invention relates to therapeutic drug delivery vehicles for ocular tissue.

Ackerman and Jones, U.S. Patent 2,923,692, describe mucilaginous compositions formed from carboxylic polymers, such as polymers of acrylic acid. These polymers are said to be useful as thickening agents. The mucilaginous compositions are said to be useful as textile printing pastes or printing inks, for cosmetics, polishing and cleaning compositions.

Carbapol has been used in some ocular delivery systems. For example, Davies et al., 9 Pharm. Res. 1137, 1992, discusses use of carbapol in phospholipid vessels for entrapment of tropicamide; and Weinreb and Tani, 46 J. Pasenter. Sci. Technol. 51, 1992, discuss use of a polyacrylic acid polymer with Betaxolol for ocular delivery.

Summary of the Invention

Applicant describes nucleic acid formulations designed for ocular delivery formed from polymers of acrylic acid. In particular, applicant describes the use of enzymatic RNA in such a formulation, useful for delivery of that enzymatic RNA to ocular tissue. In one example, an enzymatic RNA containing five 2'-O-methylated riboses in stems I and III of a hammerhead-type ribozyme (see below) were formulated in carbapol 974, which is a polymer of acrylic acid. Upon hydration with water, the polymer formed a viscous solution at 1% concentration when the pH was between 4 and 4.5. Neutralization with ammonium hydroxide resulted in the viscous solution forming a complete gel, in which the internal compartment had a high intrinsic viscosity. Addition of cation to

this gel caused the gel to collapse, thereby allowing release of the associated enzymatic RNA.

Thus, in a first aspect, the invention features a therapeutic composition containing a polyacrylic acid at a concentration sufficient to form a gel (e.g., between 0.5 and 1.5%), and a therapeutically effective amount of a nucleic acid molecule. In particularly preferred embodiments, the nucleic acid is RNA, e.g., enzymatic RNA.

Polyacrylic acids useful in the invention are described by Ackerman and Jones, *supra*, and are well known to those of ordinary skill in the relevant art. In addition, nucleic acid molecules useful for delivery are well known in the art and include enzymatic RNA molecules, or ribozymes.

Ribozymes are RNA molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence specific manner. It is said that such enzymatic RNA molecules can be targeted to virtually any RNA transcript and efficient cleavage has been achieved *in vitro*. Kim et al., 84 Proc. Natl. Acad. of Sci. USA 8788, 1987; Haseloff and Gerlach, 334 Nature 585, 1988; Cech, 260 JAMA 3030, 1988; and Jefferies et al., 17 Nucleic Acids Research 1371, 1989.

Ribozymes act by first binding to a target RNA. Such binding occurs through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA which acts to cleave the target RNA. Thus, the ribozyme first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a ribozyme has bound and cleaved its RNA target it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology

(where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

This class of chemicals exhibits a high degree of specificity for cleavage of the intended target mRNA. Consequently, the ribozyme agent will only affect cells expressing that particular gene, and will not be toxic to normal tissues.

By "enzymatic RNA molecule" it is meant an RNA molecule which has complementarity in a substrate binding region to a specified mRNA target, and also has an enzymatic activity which is active to specifically cleave that mRNA. That is, the enzymatic RNA molecule is able to intermolecularly cleave mRNA and thereby inactivate a target mRNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA to allow the cleavage to occur. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in

this invention. For *in vivo* treatment, complementarity between 30 and 45 bases is preferred.

In preferred embodiments, the enzymatic RNA molecule is formed in a hammerhead motif, but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNAseP-like RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi et al., 8 Aids Research and Human Retroviruses 183, 1992, of hairpin motifs by Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences", filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100, filed September 20, 1988; Hampel and Tritz, 28 Biochemistry 4929, 1989; and Hampel et al., 18 Nucleic Acids Research 299, 1990, and an example of the hepatitis delta virus motif is described by Perrotta and Been, 31 Biochemistry 16, 1992, of the RNAseP motif by Guerrier-Takada, et al., 35 Cell 849, 1983, and of the group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic RNA molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

By "therapeutically effective amount" is meant that amount sufficient to have an effect on the disease phenotype or condition to be treated. For example, in treatment of viral infections, such as herpes simplex virus, in ocular tissue it is the amount sufficient to lower the amount of viral nucleic acid present within the ocular tissue, or to reduce the number of viral forming units within that tissue.

In particularly preferred embodiments, the invention features administration of enzymatic RNA or ribozymes effective to treat herpes simplex virus, for example,

those molecules described by Draper, U.S. Serial No. 07/882,921, filed May 14, 1992, entitled "Method and Reagent for Inhibiting Herpes Simplex Virus Replication", Draper, U.S. Serial No. 07/948,359, filed September 18, 1992, and Draper, U.S. Serial No. 07/987,133, filed December 7, 1992, all hereby incorporated by reference herein and assigned to the same assignee as the present application.

In a related aspect, the invention features a method for treatment of ocular tissue by administering therapeutic nucleic acid to that tissue by use of a polyacrylic acid polymer, with or without an additional chelator.

The composition and methods of this invention are significantly advantageous over administration of free nucleic acid to an eye, increasing the dose delivered to an eye up to about six-fold, facilitating patient compliance by decreasing the number of required administrations, protecting the ribozyme from nuclease degradation.

The methods of this invention are also suitable for administration of ribozymes to other tissues, including lung, gut, esophagus, skin, nasal, vaginal and even veins.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a graphical representation showing ocular delivery of ribozymes using unpolymerized carbapol 974.

Fig. 2 is a histogram showing ribozyme uptake by ocular tissue in relationship to percent carbapol 974.

Fig. 3 is a copy of various autoradiograms showing retention of ribozymes by ocular tissue after delivery in water, or in polymerized or unpolymerized carbapol 974.

Fig. 4 is a copy of various autoradiograms showing degradation of delivered ribozymes by micrococcal nuclease.

Fig. 5 is a dose response curve showing total amount of ocular associated ribozyme prior to extraction (B), and the amount of ribozyme extracted from eye (C). (Ribozyme extraction efficiency was 50%.)

Polyacrylic Acid

Polyacrylic acids useful in the present invention are generally described in the art cited above. Below are provided examples of use of various polyacrylic acids as ocular delivery vehicles for ribozymes and other nucleic acids. Specifically, five forms of polyacrylic acid were obtained from B.F. Goodrich, including carbapol 974, Pemulens TR1 and TR2, and carbapol 940. Prior to testing *in vivo* in mice, *in vitro* studies were performed to determine the compatibility of these polymers with the nucleic acid. These examples are not limiting in the present invention, but are provided merely to illustrate to those of ordinary skill in the art the breadth of the invention. While ribozymes exemplify the use of nucleic acid for delivery, they are not limiting in the invention.

Applicant has found that these polyacrylic acids are useful for topical delivery, e.g., ocular, buccal or vaginal delivery, of nucleic acids. Unpolymerized polyacrylic acid including the nucleic acid is a fluid, but when placed in contact with cationic groups or neutral pH (as found in tears) it forms a gel and allows slow release of the nucleic acid. Thus, it forms a useful pharmaceutical delivery vehicle composition. The following examples illustrate these properties and the use of such polyacrylic acids.

Example 1: Ribozyme/Polyacrylic Acid Mixtures

Carbapol is a polymer of acrylic acid and has some useful properties as an ocular delivery vehicle. Upon hydration with water, a viscous solution forms at 1% concentration when the pH is between 4 and 4.5 (unpolymerized carbapol). Neutralization with ammonium hydroxide results in the viscous solution forming a complete gel, in which the internal compartment has a high intrinsic viscosity (polymerized carbapol). Addition of a cationic moiety causes the gel to collapse. This was demonstrated by adding a hammerhead ribozyme (any one can be used) to the acid solution of the polymer on a piece of parafilm. Addition of water washed the ribozyme off the parafilm, as did addition of ammonium hydroxide followed by a phosphate buffered saline wash (150 mM NaCl, PBS). Addition of ammonium hydroxide followed by the water wash, however, did not wash the ribozyme off the parafilm. The same results were observed when a collagen shield was substituted for the parafilm.

Specifically, carbapol dry powder was hydrated with an aqueous phase containing a ribozyme. The concentration of ribozyme was 10 mg/ml and the carbapol was hydrated to 0.5%. Upon hydration, the viscous solution had a pH between 3 and 4. The solution was polymerized by bringing the pH to 7 with 0.2 M NH_4OH. The degree of ribozyme association was determined by layering 1 ml 30% ficoll 400 in water over the polymerized gel, followed by 1 ml 10% ficoll with 1 ml water layered over the top. The tubes were centrifuged and the polymerized gel with the ribozyme floated to the top. 75% of the ribozyme was at the 10% ficoll/water interface. In the absence of carbapol, no ribozyme was located at the 10% ficoll/water interface.

In another method, which was essentially the same as above, the same amount of ribozyme was located at the 10% ficoll/water interface when the ribozyme was added to a solution of carbapol, rather than hydrating the dry powder with a solution of ribozyme.

The carbapol hydrated with ribozyme, or a 1/1 solution of 1% carbapol and 1 to 2 mg/ml ribozyme, were added to a piece of parafilm. The gel was polymerized by the addition of ammonium hydroxide. The strip of parafilm was washed in water 3X to remove any non-polymer associated ribozyme. The strips were washed with phosphate buffered saline (0.8% NaCl, 0.02% KCl and 10 mM Na phosphate), pH 7.4, and the strips were counted. 100% of the ribozyme was retained on the parafilm after 3x water wash. 0% remained after 1x wash with PBS.

Example 2: Ocular Retention of Delivered Ribozyme

The following procedure was used to measure ribozyme delivery to ocular tissue. Mice were anesthetized with sodium pentobarbital with a dose of 50 mg/kg. Ten μg of a hammerhead-type ribozyme was enzymatically labeled with $^{32}P\gamma$-ATP at the 5' end. The radiolabeled ribozyme was used to spike cold ribozyme yielding a specific activity between 7.5 X 10^4 and 1.5 X 10^5 DPM/μg. The ribozyme was mixed 1:1 with a 1% unpolymerized carbapol solution in water. A dose of 0.5 μg, 1 μg, 5 μg, 10 μg and 20 μg was applied to the mouse eye in 0.5% carbapol in a volume of 2 μl. The animals were euthenized at 3, 10, 30 and 60 minutes after administration. The eyes were surgically removed, washed in 10 ml PBS 3X, added to 200 μl lysis buffer [4M guanidinium isothiocynate 0.5% sarcosyl 25 mM sodium citrate] and immediately frozen in liquid nitrogen. The eyes were thawed by sonication in a strong bath sonicator (Scientific Products, Hicksville, Long Island). The freezing and bath sonicator thawing was repeated 2 more times. The total RNA from the eyes was extracted using a chloroform/phenol extraction procedure "Guanidinium Methods for Total RNA Preparation," Current Protocols in Molecular Biology, (Ed. F.M. Ausubell) Greene Publishing Assoc., and Wiley-Interscience, John Wiley & Sons, New York, pp. 4.2.1-4.2.8, (1992). The ethanol precipitated RNA was re-suspended in 10 μl gel loading

buffer [95% formamide, 20 mM EPTA, 0.5% xylene cyanol, 0.05% bromophenol blue]. Two μl was counted for radioactivity and 8 μl applied to a 20% acrylamide gel.

The following studies were performed with a hammerhead ribozyme that contains five 2'-O-methyl riboses in stem I and stem III of the ribozyme:

1. The optimal percent of carbapol 974 was determined using the above protocol, and applying 1 μg of ribozyme to each eye in 0.0%, 0.05%, 0.10% and 0.5% carbapol. The results are summarized in Fig. 2. 0.5% carbapol was selected to be the optimum. No higher concentration of carbapol was tested because the solution was too viscous, and reproducability of dose delivery was compromised.

2. A time course for retention of intact ribozyme was performed using carbapol 974. One μg of the radiolabeled ribozyme was applied to the eye with 0.5% carbapol 974, and the eyes were removed 3, 10, 30 and 60 minutes after application. Two mice were used per time point. The results are shown in Fig. 1. The results showed that an optimum eye retention was obtained 30 minutes after administration, yielding 4 pmol retained by the eye from an 83 pmol dose.

3. A dose response curve was tested from 0.5 μg to 20 μg (42 pmol to 1660 pmols) ribozyme. The ribozyme was administered with 0.5% carbapol in a volume of 2 μl. The results show the uptake was linear over the dose range tested.

The above experiments show that 5% of the administered dose is retained by the eye after 3 minutes and 10 minutes topical administration to the mouse eye. Free ribozyme yields 1% retention. We also found that unpolymerized carbapol-containing ribozyme yields a higher percentage of uptake, compared to administration of polymerized carbapol-containing ribozyme.

Example 3: Ocular Retention

To determine if the ribozyme was in the eye as opposed to being bound to the outside, a comparison of washes was made. The mouse eyes were washed with water, PBS or PBS + micrococcal nuclease. The PBS wash removed 80% of the ribozyme from the polymerized carbapol, but only 20% from the unpolymerized carbapol. The amount of ribozyme administered without carbapol was unchanged. The micrococcal nuclease treatment showed a 40% reduction in the amount of ribozyme retained by the eye. However, it was observed that the carbapol inhibited the micrococcal nuclease activity. This may have been due to a variety of reasons, but one control that was run was to inhibit the enzyme by the addition of EDTA. Carbapol is composed of acrylic acid monomers, which has a terminal carboxy group capable of binding mono-, di- and tri- valent cations. Thus, the inhibition may be the result of the carbapol chelating the required divalent co-factor. An advantage of this observation is that the ribozyme complexed with carbapol is resistant to nuclease degradation.

Specifically, the following experiment was designed to determine if the retained ribozyme was intracellular or just bound to the surface of the eye. The study used 1 μg of a hammerhead-type ribozyme with 0.5% carbapol 974. Time points were taken 3 and 10 minutes after administration and two mice were used per time point. Free ribozyme was compared to carbapol 974 in the polymerized and unpolymerized form. To polymerize the gel, 1 μl of ribozyme and 1 μl of 1% carbapol were mixed on a piece of parafilm. Addition of 1 μl of 0.2 M NH_4OH caused the mixture to polymerized gel. This was then applied to the eye as a smear. The eyes were washed in water, PBS and PBS followed by treatment with micrococcal nuclease treatment for 1 minute. Addition of PBS to carbapol caused the collapse of the gel and release of the ribozyme as shown in *in vitro* experiments. Other results

showed that within this time frame, the micrococcal nuclease degraded 50% of 1 μg ribozyme.

The results are shown in Fig. 4. The top panel (A) is free ribozyme, the middle panel (B) is unpolymerized carbapol, and the bottom panel (C) is polymerized carbapol. The results show that the carbapol yielded higher retention of the ribozyme compared to free ribozyme. Furthermore, the unpolymerized carbapol gave better retention of the ribozyme than the polymerized carbapol. The fact that less was removed by the PBS wash indicates that the ribozyme may be intracellular.

Ocular Tissue Localization of Carbopol/Ribozyme

Ocular fixation followed by tissue sectioning was used to determine the tissue distribution of the ribozyme within the eye. The ribozyme was labeled at the 5′ end with ^{33}P. This isotope is a low energy b-emitter which produces discrete silver grains when exposed to photographic film. 1 μg of ribozyme was applied to the eye in 0.5% cabopol at acidic pH. At 10 minutes and 30 minutes after administration, the mice were euthanized, the eyes were removed and washed. One eye was fixed in glutaraldehyde or frozen, and the ribozyme was extracted from the other eye and analyzed by gel electrophoresis. The fixed eyes were cut into 8 μm sections and exposed to a photographic emulsion. Silver grains were over the epithelium and stromal layers of the cornea. The density of the grains decreased from the outermost layer to the innermost layer. These results showed that the ribozyme was not just sticking to the outer surface of the eye but was penetrating into the cells of the tissue.

Pharmaceutical Compositions

The above formulations can be administered as noted above, or co-formulated with standard pharmaceutically acceptable carriers or diluents described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co.

(A-G, Gennaro ed., 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers and dyes may be provided. Id at 1449. In addition, antioxidants and suspending agents may be used. Id.

Other embodiments are within the following claims.

Claims

1. Pharmaceutical composition comprising polyacrylic acid and a therapeutically effective amount of a nucleic acid.

2. The pharmaceutical composition of claim 1, wherein said nucleic acid is an enzymatic RNA molecule.

3. The pharmaceutical composition of claim 2, wherein said RNA molecule is in a hammerhead motif.

4. The pharmaceutical composition of claim 3, wherein said RNA molecule is in a hairpin, hepatitis Delta virus, group I intron, or RNAseP RNA motif.

5. The pharmaceutical composition of claim 3, wherein said ribozyme comprises between 30 and 45 bases complementary to said mRNA.

6. The pharmaceutical composition of claim 1, wherein said polyacrylic acid is not polymerized.

7. Method for delivery of a nucleic acid comprising administering the pharmaceutical composition of claim 1.

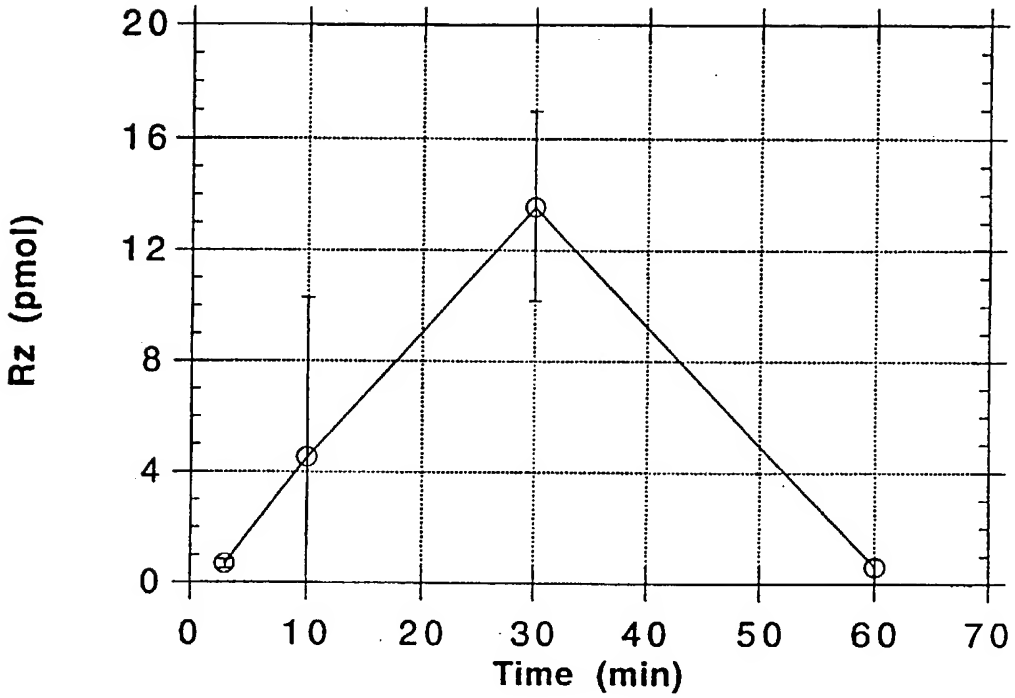


FIG. 1

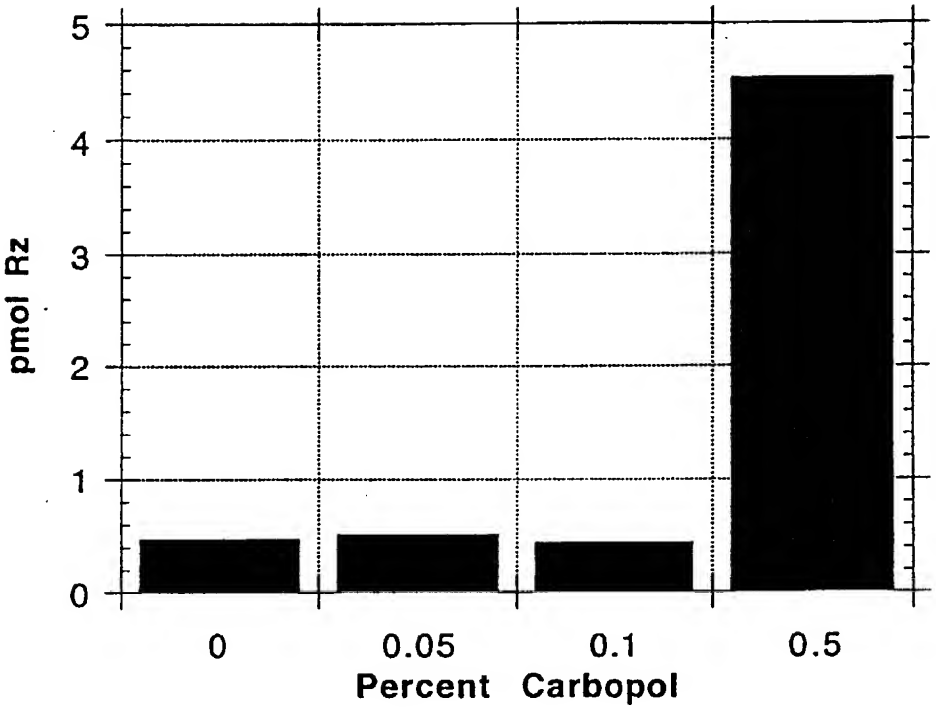


FIG. 2

FIG. 3a.

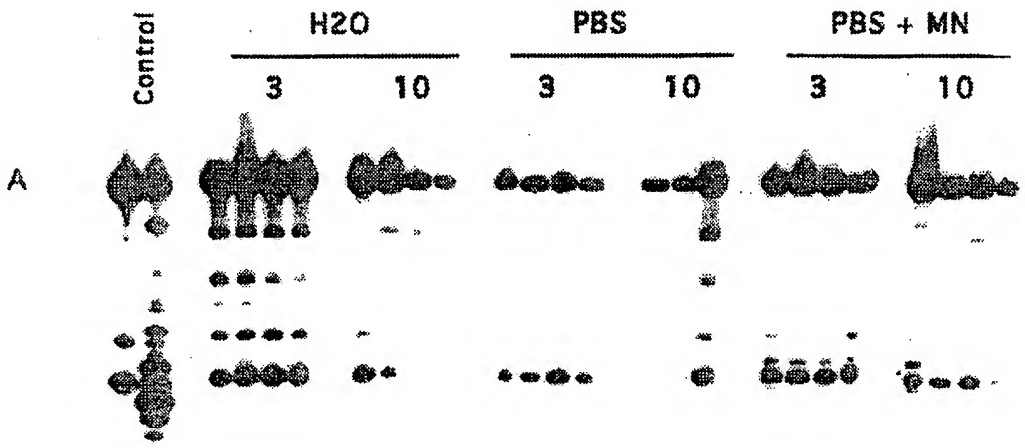

Control
H2O
PBS
PBS + MN
3
10
3
10
3
10
A

FIG. 3b.

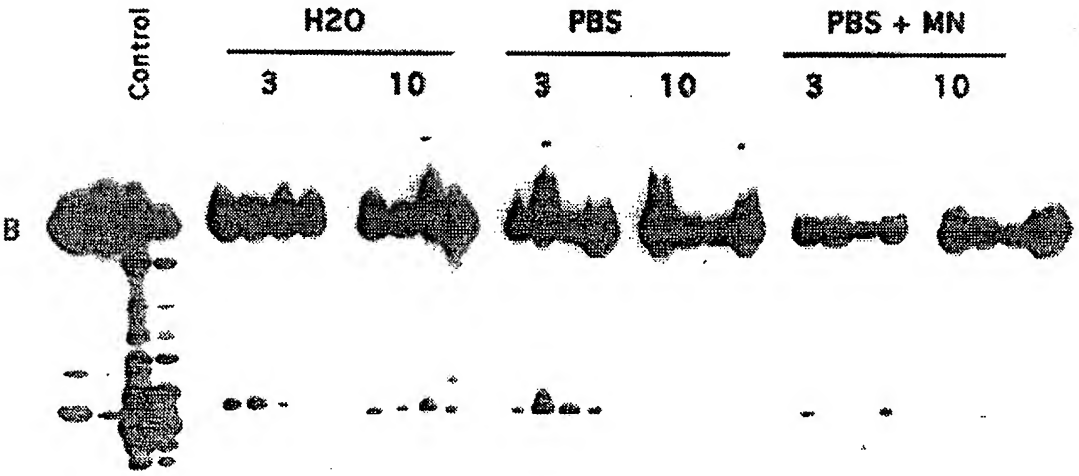

Control
H2O
PBS
PBS + MN
3
10
3
10
3
10
B

FIG. 3c.

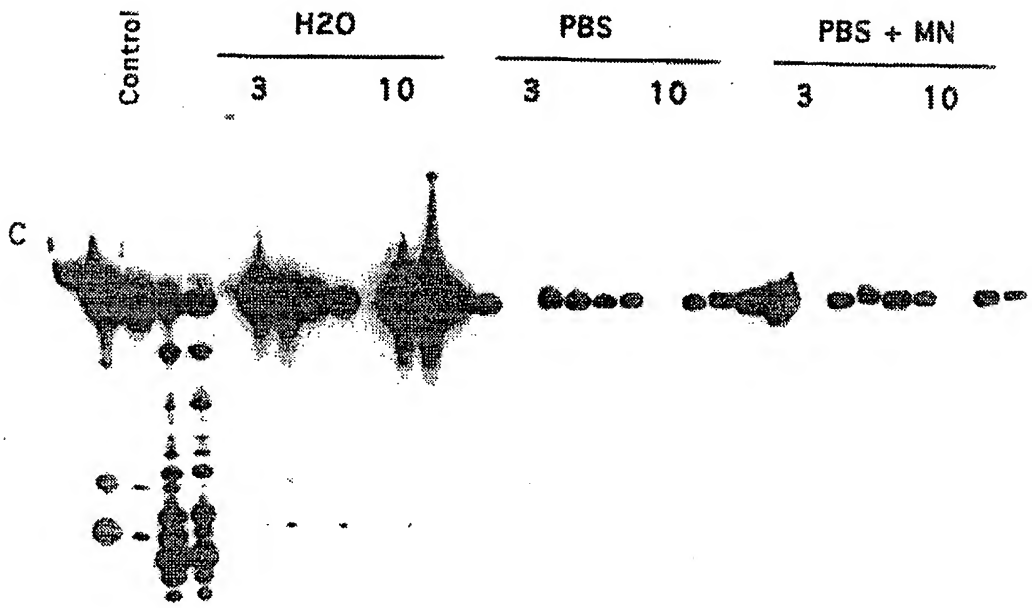


FIG. 4.

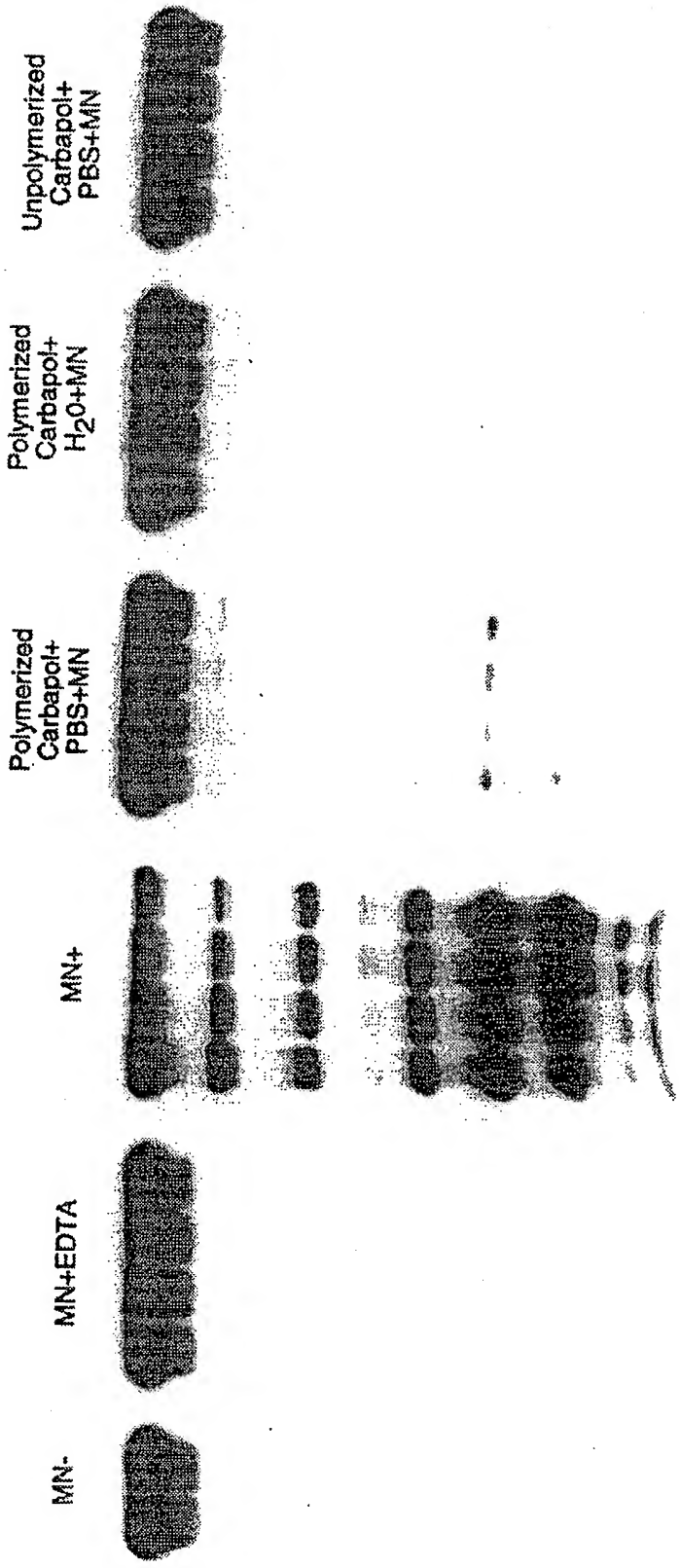
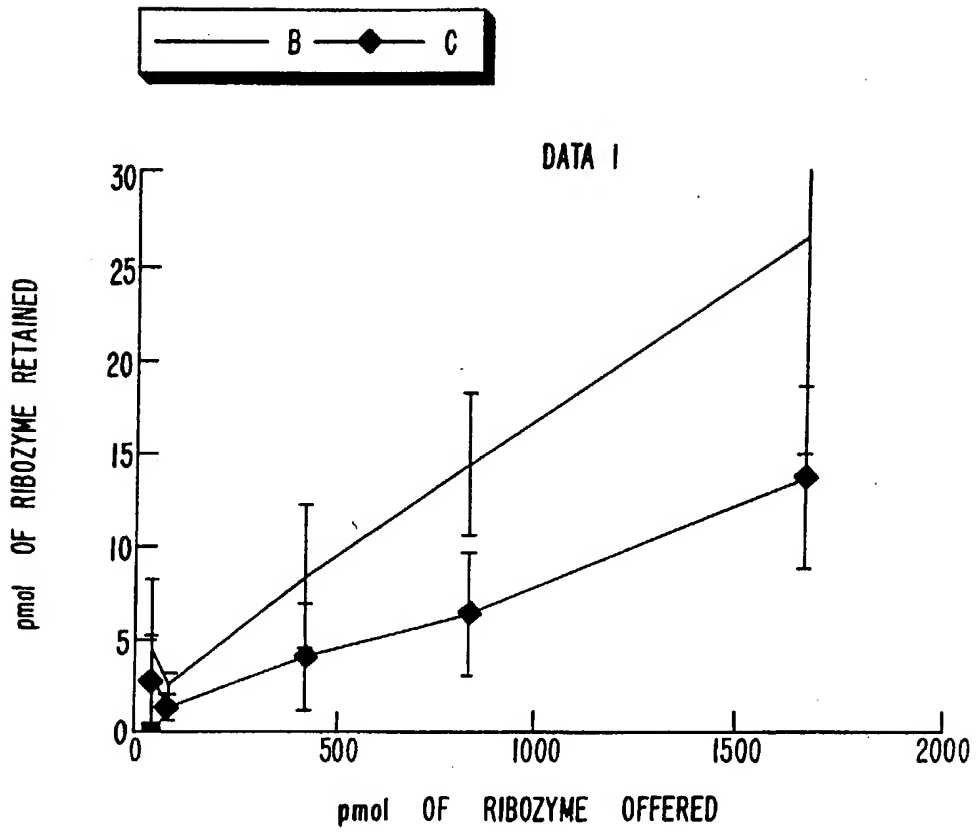


FIG. 5



B IS UNEXTRACTED Rz
C IS UNEXTRACTED Rz
Rz EXTRACTION EFFICIENCY FROM THE EYE IS 50 %